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(54) Title: EXPRESSION OF PROTEINS FROM AMPLIFIED, IMMOBILIZED NUCLEIC ACIDS

(57) Abstract: A protein is made by immobilizing a PCR primer onto a solid support, then using the immobilized PCR primer along with a soluble primer to amplify and immobilize a template DNA containing a protein coding sequence onto the solid support. The template DNA and/or the PCR primers also contain regulatory sequences for transcription and translation of the coding sequence. The immobilized DNA is then transcribed and translated to produce the protein. The immobilized DNA may be reused for multiple cycles of transcription and translation. By immobilizing a universal PCR primer onto the solid support, different template DNAs can be amplified and immobilized and a number of different proteins can be made at the same time. If particles are used as the solid support, the particles may be injected into an organism so that the steps of transcription and translation take place within the organism to produce a protein vaccine or therapeutic.



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## 1      **EXPRESSION OF PROTEINS FROM AMPLIFIED, IMMOBILIZED NUCLEIC ACIDS**

### 3                      **Technical Field**

4              The present invention relates to the production of proteins generally and in particular to the  
5      production of proteins using DNA that is immobilized and amplified onto a solid surface by means  
6      of an immobilized oligonucleotide primer.

### 8                      **Background Art**

9              The polymerase chain reaction (PCR) is a powerful tool that enables logarithmic  
10     amplification of DNA sequences, resulting in a  $10^6$  -  $10^8$  fold enhancement of a target sequence. The  
11     amplified DNA can be used for a variety of downstream experimental or diagnostic purposes, such  
12     as the detection of a minute amount of a particular DNA sequence in a sample. The basics of PCR  
13     are described, for example, in the following U.S. patents: U.S. Patent No. 4,683,195 to Mullis et al  
14     for "Process for Amplifying, Detecting and/or Cloning Nucleic Acid Sequences", U.S. Patent No.  
15     4,683,202 to Mullis for "Process for Amplifying Nucleic Acid Sequences" and U.S. Patent No.  
16     4,800,159 to Mullis et al for "Process for Amplifying, Detecting and/or Cloning Nucleic Acid  
17     Sequences".

18             As efficient as the PCR process can be, recovery of amplification products for subsequent  
19     uses can involve tedious purification procedures such as gel electrophoresis, organic extraction,  
20     centrifugation, and/or column purification. Consequently, methods have been developed for creating  
21     immobilized PCR amplicons by immobilizing a PCR primer onto a solid substrate. Such methods  
22     are described, for example in the following patents and publications: Kohsaka et al "Solid-Phase  
23     Polymerase Chain Reaction", Journal of Clinical Laboratory Analysis 8 (1994), pp 452-455;  
24     Rasmussen et al, "Combined Polymerase Chain Reaction-Hybridization Microplate Assay Used to  
25     Detect Bovine Leukemia Virus and *Salmonella*" Clinical Chemistry, vol. 40, No. 2 (1994), pp 200-  
26     205; Oroskar et al, "Detection of Immobilized Amplicons by ELISA-like Techniques" Clinical  
27     Chemistry vol. 42, No. 9 (1996) pp 1547-1555; Lockley et al "Colorimetric Detection of  
28     Immobilised PCR Products Generated on a Solid Support" Nucleic Acids Research vol 25, No. 6  
29     (1997) pp 1313-1314; U.S Patent No. 5,922,574 to Minter for "Method for Producing Copies of a  
30     Nucleic Acid Using Immobilized Oligonucleotides"; and U.S. Patent No. 5,910,406 to Minter for  
31     "Manipulating Nucleic Acid Sequences". Typically, immobilized PCR primers are used in  
32     diagnostic assays to detect the presence of minute amounts of specific DNAs in a sample.

1 In a separate field of technology, methods have been developed for *in vitro* synthesis of  
2 proteins by cell-free transcription and translation of protein-encoding DNA. Cell-free synthesis of  
3 proteins is disclosed, for example, in the following U.S. patents: U.S. Patent No. 5,556,769 to Wu  
4 et al for "Coupled Replication-Translation Methods and Kits for Protein Synthesis"; U.S. Patent No.  
5 5,492,817 to Thompson et al for "Coupled Transcription and Translation in Eukaryotic Cell-Free  
6 Extract"; U.S. Patent No. 5,665,563 to Beckler for "Coupled Transcription and Translation in  
7 Eukaryotic Cell-Free Extract"; and U.S. Patent No. 5,895,753 to Mierendorf et al for "Method for  
8 In Vitro Protein Synthesis". Coupled methods of DNA amplification and protein synthesis are  
9 described, for example, in the following U.S. Patents: U.S. Patent No. 5,571,690 to Hecht for  
10 "Method for the Cell-Free Synthesis of Proteins" and U.S. Patent No. 5,807,717 to Joyce for  
11 "Coupled Isothermal Polynucleotide Amplification and Translation System". Typically, the  
12 amplified DNA used in the production of the protein is not recoverable or reusable, but rather is  
13 discarded during the process of purifying the protein.

14 A method for producing RNA from immobilized DNA templates is described in U.S. Patent  
15 No. 5,700,667 to Marble et al for "Strategy for the Production of RNA from Immobilized  
16 Templates".

17 In another area of technology, DNA vectors have been developed for genetic vaccines and  
18 genetic therapeutics. In a technique known as genetic immunization, a DNA vector containing a  
19 coding sequence for a protein antigen as well as appropriate regulatory sequences is injected into an  
20 organism. Immunization can be by way of intramuscular, intravenous or intra-nasal routes. Inside  
21 the organism, the DNA is transcribed into RNA and the RNA is translated into the protein antigen.  
22 The protein antigen then triggers an immune response in the organism, typically activating both the  
23 humoral and cellular immune responses. Methods of genetic immunization are described, for  
24 example, in the following U.S. patents: U.S. Patent No. 5,589,466 to Felgner et al for "Induction of  
25 a Protective Immune Response in a Mammal by Injecting a DNA Sequence"; U.S. Patent No.  
26 5,739,118 to Carrano et al for "Compositions and Methods for Delivery of Genetic Material"; U.S.  
27 Patent No. 5,795,872 to Ricigliano et al for "DNA Construct for Immunization"; U.S. Patent No.  
28 5,814,617 to Hoffman et al for "Protective 17 KDA Malaria Hepatic and Erythrocytic Stage  
29 Immunogen and Gene"; U.S. Patent No. 5,830,876 to Weiner et al for "Genetic Immunization"; and  
30 U.S. Patent No. 5,817,637 to Weiner et al for "Genetic Immunization". In genetic therapy, a DNA  
31 construct with appropriate regulatory and protein coding sequences is injected into an organism to  
32 produce a therapeutic protein. Typically, the purification steps required with present methods of

1 production of DNA for gene vaccines and gene therapy are extensive and time-consuming. These  
2 approaches may be subject to great scrutiny by the FDA due to the presence of xenobiotic (e.g.  
3 bacterial or viral) DNA, and the possible presence of endotoxins resulting from plasmid synthesis  
4 in bacteria.

#### 6 **Disclosure of Invention**

7 It has now been discovered the steps of transcription and translation in protein synthesis can  
8 be carried out using DNA (containing the necessary coding and regulatory sequences) that is  
9 immobilized on a solid support. Further, it has been discovered that the method by which the DNA  
10 is immobilized onto the solid support has a dramatic effect on the efficiency of thermal cycling  
11 amplification as well as subsequent transcription and translation reactions. Immobilized DNA that  
12 has a density and a thermal stability optimized for amplification and subsequent transcription and  
13 translation reactions can be created by first covalently attaching an oligonucleotide primer, such as  
14 a PCR primer, to a solid support and then using the oligonucleotide primer in an amplification  
15 reaction, such as the polymerase chain reaction, to amplify the desired DNA. Additional stability  
16 may be gained by incorporating non-natural chemistries such as phosphorothioate linkages in the  
17 terminal sequences of at least one of the second oligonucleotide primers.

18 Accordingly, the present invention is directed to a method of making a protein comprising  
19 the steps of:

20 (a) providing

21 (i) a solid support,

22 (ii) a template DNA that includes at least one sequence that encodes a protein, and

23 (iii) first and second oligonucleotide primers, wherein the first oligonucleotide  
24 primer, the second oligonucleotide primer and/or the template DNA include at least  
25 one transcription regulatory sequence and at least one translation regulatory  
26 sequence, and wherein the first oligonucleotide primer is covalently attached to the  
27 solid support,

28 (b) amplifying the template DNA using the first and second oligonucleotide primers to  
29 obtain amplicons covalently attached to the solid support, wherein the amplicons include the  
30 transcription and translation regulatory sequences and the protein coding sequence,

31 (c) transcribing the covalently attached amplicons to obtain an RNA segment that includes  
32 at least one protein coding sequence and at least one translation regulatory sequences, and

1 (d) translating the RNA segment to obtain the protein.

2 The present invention further includes a device for making a protein, the device comprising  
3 (i) a solid support, and (ii) a DNA segment having a first end and a second end, wherein the first  
4 end is covalently attached to the solid support, wherein the second end contains phosphorothioate  
5 linkages, and wherein the DNA segment includes at least one protein coding sequence, at least one  
6 transcription regulatory sequence and at least one translation regulatory sequence.

7 The method of synthesis of proteins using immobilized DNA according to the present  
8 invention has advantages over methods of synthesis of proteins using unbound DNA in that the  
9 present method allows for DNA to be separated from a reaction mixture after the synthesis is  
10 completed and to be reused in subsequent rounds of transcription and translation. Separation can  
11 be easily accomplished by washing or, if the DNA is immobilized onto particles, by centrifugation  
12 or by magnetic separation. Separation and reuse of DNA is much less practicable if DNA is not  
13 immobilized on a solid support. Another advantage of the present invention is that the step of  
14 obtaining an amplified, protein-coding sequence is simplified by the approach of using an  
15 immobilized primer to simultaneously amplify and capture the amplicon, which eliminates many  
16 purification steps. Still another advantage of the present invention is that multiple amplicons can be  
17 amplified and immobilized from a single PCR reaction, and from these, multiple proteins may be  
18 simultaneously prepared. Still another advantage is that the beads may be derivatized to target  
19 immobilized DNA sequences to specific cell types or tissues.

20 The method of immobilizing DNA onto a solid support by covalently attaching a PCR primer  
21 and then extending the primer through the polymerase chain reaction has been found by the  
22 inventors to provide a more favorable attachment of DNA than a method of first amplifying DNA  
23 and then attaching the amplified DNA to a solid support. Moreover, for carrying out the method of  
24 the invention, solid supports with "universal" PCR primers (that is, primers that recognize a variety  
25 of template targets) can be prepared in advance.

26 A further aspect of the invention is that DNA that is covalently attached to particles could  
27 be injected into an organism as a genetic vaccine or genetic therapeutic. Transcription and  
28 translation to produce a protein would then take place within the organism. It is expected that  
29 coding and regulatory sequences of DNA that is covalently attached to particles will stand a better  
30 chance of being transferred intact into an organism than naked DNA or DNA that is merely  
31 physisorbed onto particles. It is also expected that DNA prepared according to the method described  
32 by the inventors will be composed of minimal xenobiotic (to human) DNA and minimal endotoxin

1 contamination. DNA that is covalently attached to particles can be protected from enzymatic  
2 degradation in an organism by incorporating chemically modified internucleotidic linkages such as  
3 phosphorothioate into the second PCR primer. This is not possible with conventional plasmid  
4 techniques.

5 Another advantage of the present invention is that it provides a way to synthesize several  
6 different proteins at one time in a single reaction mixture. This may be done, for example, by  
7 immobilizing several different PCR primers to the solid support and then amplifying several  
8 different template DNAs onto the solid support and transcribing and translating each of these  
9 simultaneously. It may also be done by selecting a first PCR primer that recognizes several different  
10 template DNAs, then simultaneously amplifying and immobilizing the several different DNAs onto  
11 the solid support, then transcribing and translating each of these simultaneously. If particles such  
12 as beads are used as the solid support, different template DNAs could be amplified separately onto  
13 particles and then the different particles could be combined into a single reaction mixture for  
14 transcription and translation.

#### 17 **Best Modes for Carrying Out the Invention**

18 As used herein, the term "solid support" refers to any surface or particle to which  
19 oligonucleotides may be attached. Preferably, the solid support is in the form of particles, such as  
20 glass particles, controlled porous glass particles, magnetic particles, polymeric particles or ceramic  
21 particles. Preferably, the particles are small enough so that they can be kept suspended in an  
22 aqueous solution with gentle agitation and are dense enough so that they can be easily recovered  
23 from a reaction medium. Typically, the particles will have a particle size of about 50 nanometers  
24 to 10 mm along an axis of greatest length. The particles may be any shape or form, including  
25 spherical or irregular three-dimensional shapes or two-dimensional shapes. Commercially available  
26 controlled porous glass beads or polymer beads such as are commonly used for solid phase organic  
27 synthesis are suitable as solid supports for the present invention.

28 The solid support may also be a portion of the surface of a membrane, filter, test tube,  
29 microtiter well, microscope slide or other surface or may be the surface of a material such as a  
30 hydrogel or sol-gel.

31 As discussed below, the solid support may include any surface modification that enables the  
32 first oligonucleotide primer to be covalently attached to the solid support.

1           As used herein, the term "protein" refers to any peptide or polypeptide that can be produced  
2 by the expression of DNA. The term includes complete functional proteins such as enzymes,  
3 hormones and antibodies, etc., fragments of complete proteins, fusions of two or more complete  
4 proteins or fragments, and fusions of complete proteins or fragments with additional peptide  
5 sequences.

6           As used herein, the term "template DNA" refers to a segment of DNA that includes a  
7 nucleotide sequence that encodes a protein and may also include regulatory sequences operatively  
8 linked for directing the expression, including transcription and translation, of the coding sequence.  
9 The template DNA may be produced by DNA synthesis or derived from expression vectors,  
10 plasmids, cosmids, genomic DNA or RNA (or a subset thereof), PCR amplicons, amplicons derived  
11 from other enzymatic or non-enzymatic means, intact or lysed bacteria, viral plaques, or cells. The  
12 particular coding sequence is, of course, selected according to the particular protein that is to be  
13 made. The regulatory sequences are selected according to the particular expression system (bacterial,  
14 mammalian, insect, yeast, etc.) to be used for expressing the DNA. Examples of transcriptional  
15 regulatory sequences include promoters for T7 RNA polymerase, SP6 RNA polymerase,  
16 cytomegalovirus, adenovirus, adenoassociated virus, lentiviruses, poxviruses, baculovirus and yeasts  
17 such as *Saccharomyces*. It is not necessary, however, that entire regulatory sequences be contained  
18 in the template DNA. Some regulatory sequences necessary for expression may be located initially  
19 in the first PCR primer or the second PCR primer. These regulatory sequences would then become  
20 operatively linked to the coding sequences when the first oligonucleotide primer and second  
21 oligonucleotide primer are extended in the nucleic acid amplification reaction to form the  
22 immobilized amplicons.

23           As used herein, the terms "first PCR primer" and "second PCR primer" refer to  
24 oligonucleotides that are selected according to conventional PCR techniques to hybridize on  
25 opposite ends and opposite strands of the template DNA. As described below, the first PCR primer  
26 is covalently attached to a solid support (and is also provided in low concentration as a soluble first  
27 primer) and the second PCR primer is soluble. By referring to "PCR" primers, it is not meant to limit  
28 the method of the invention to amplification by PCR in its strictest and most precise definition. As  
29 used herein, the terms "first PCR primer", "second PCR primer" and "polymerase chain reaction"  
30 can be used interchangeably with the terms "first oligonucleotide primer", "second oligonucleotide  
31 primer" and "amplification reaction" to refer to PCR or similar methods that use primer recognition  
32 of a template DNA and primer extension to achieve DNA amplification or ligation of two or more

1 nucleic acid sequences.

2 Both the first PCR primer and the second PCR primer may contain additional sequences  
3 besides those that are strictly necessary to effect a PCR amplification of the template DNA, and  
4 these additional sequences would, during the PCR process, be incorporated into the immobilized  
5 amplicons. For example, the first PCR primer or the second PCR primer may contain regulatory  
6 sequences that affect the transcription and translation of the coding sequences of the template DNA.  
7 The second PCR primer may contain modifications to its 5' end to make the amplicon more resistant  
8 to enzymatic degradation. For example, the 5' end of the second PCR primer may include  
9 phosphorothioate or methyl phosphonate-modified nucleotides.

10 In the Examples described below, the bacteriophage T7 transcriptional promoter and  
11 termination sequences were used as primers since the T7 promoter allows for high levels of tightly  
12 regulated transcription and thus may be useful for recombinant cloning and protein production  
13 applications. See, for example, Fujita, K., and Silver, J. (1993) *Biotechniques*, 14, 608-617. With  
14 design of appropriate primers, however, it is anticipated that this method would work equally well  
15 for other bacterial, viral or mammalian expression systems.

16 For the steps of solid stage amplification and transcription to be carried out efficiently and  
17 effectively, the first PCR primer must be coupled to the solid support with a bond that is stable  
18 enough to hold up through the process of thermal cycling during PCR amplification. Covalent  
19 bonding of the first PCR primer to the solid support is required. In particular, it has been discovered  
20 in the present invention that many currently known methods of attaching oligonucleotides to a solid  
21 support are insufficient to provide a stable enough attachment. For example, a method of covalent  
22 attachment of DNA to a solid support described by Chrisey, et al, *Nucleic Acids Research* (1996),  
23 24, 3131-3039 and US Patent 5,688,642 to Chrisey, et al, utilized a sulfhydryl-terminated  
24 oligonucleotide to react with an aminated surface which had been treated with a hetero-bifunctional  
25 crosslinker containing N-hydroxysuccinimide and iodoacetamide groups, to produce a thioether  
26 linkage. This covalent attachment scheme was not sufficiently heat stable to be useful for an  
27 amplification strategy that depends on thermal cycling. Another method that was sub-optimal  
28 utilized a commercially available "oligo affinity" DNA synthesis support, which is designed to allow  
29 synthesis of a DNA sequences onto a support from which it cannot be readily cleaved. This  
30 approach produced sequences that lacked fidelity through normal levels of synthesis failure;  
31 moreover, the density of the immobilized primers was sub-optimal.

32 For extremely long amplicons, such as amplicons with more than 5000 bases, it may be



1 desirable to prepare the amplicon separately, purify it, and then couple to a solid support in  
2 preparation for transcription/translation, due to the difficulty, low yields and special conditions  
3 required for fidelity in amplifying such sequences.

4 Preferably, to create a heat-stable attachment of the first PCR primer with the solid support,  
5 the solid support is first modified with a silane layer that includes available reactive amine groups.  
6 For example, the surface of the support may be silanized using N-(2-aminoethyl)-3-  
7 aminopropylmethyl-dimethoxy-silane (EDA, Gelest, Tullytown, PA) or N-(6-aminoethyl)-  
8 aminopropyltrimethoxysilane (HDA, Gelest). The first PCR primer may also be modified to  
9 include reactive amine or phosphate groups. The solid support may then be coupled to the first PCR  
10 primer by carbodiimide chemistry. For example, a phosphorylated primer may be coupled to an  
11 amino-functionalized surface using 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide-HCl (EDC,  
12 Sigma Chemical Co, St. Louis, MO) in a 1-methylimidazole (Sigma) buffer to generate an amide-  
13 coupled primer.

14 The covalent attachment of the first PCR primer to the solid support may also be achieved  
15 by synthesizing the primer directly on the support by automated DNA synthesis using a non-  
16 cleavable synthesis support.

17 Because the steps of PCR amplification and transcription require the accessibility of enzymes  
18 and reactants to the individual molecules of the first PCR primer, it is preferable that the first PCR  
19 primer be attached to the solid support by means of a tether or spacer such as a heterobifunctional  
20 or homobifunctional crosslinker. The crosslinker is selected based on the specific chemical  
21 functionalities of the surface and the primer to be tethered. To attach an oligonucleotide to a solid  
22 surface by means of a crosslinker, the most reactive end of the crosslinker is generally reacted first.  
23 For example, crosslinkers bearing a succinimide ester are susceptible to hydrolysis, thus this end of  
24 the crosslinker would be reacted with an amine-functionalized surface or DNA first, to maximize  
25 crosslinking. The crosslinker is selected to obtain a thermally- and chemically stable linkage, such  
26 as an amide bond, while avoiding those which would generate disulfides or thioethers. The  
27 synthesis of DNA is so flexible almost any reactive group can be stably incorporated. Other design  
28 considerations in selection of crosslinker are length and flexibility, which may influence the  
29 accessibility of the DNA to enzymes and nucleotides involved in transcription.

30 The step of amplifying the template DNA by the polymerase chain reaction using the first  
31 and second PCR primers to obtain amplicons covalently attached to the solid support may be carried  
32 out by any known method of PCR or similar methods, including enzymatic and non-enzymatic

1 methods, methods that involve thermal cycling and methods that are isothermal. It is to be  
2 understood that the step is carried out in the presence of any reagents and materials useful or  
3 necessary for carrying out PCR including, for example, deoxynucleotide triphosphates, enzymes,  
4 buffer salts, and proteins such as BSA or gelatin. The reaction medium may also include a soluble  
5 version of the first PCR primer to act as a spike in promoting the PCR reaction.

6 As used herein, the term "amplicon" refers to the entire stretch of DNA that is immobilized  
7 onto the solid surface, including sequences contained in the first PCR primer, the second PCR  
8 primer, the template, as well as any sequences that may be attached to the solid surface before the  
9 first PCR primer is attached. However, the term "transcribing the covalently attached amplicons"  
10 is not meant to imply that the entire length of the amplicon is transcribed. The portion of the  
11 amplicon that is transcribed is a section that contains the coding sequence and the translation  
12 regulatory sequence. The selection of the portion of the amplicon to be transcribed is typically  
13 governed by the transcription regulatory sequence

14 If particles are used as the solid support, then following the amplification step, the particles  
15 may be recovered by separation techniques including, but not limited to centrifugation, filtration,  
16 magnetic separation, or gravity separation.

17 The step of transcribing the immobilized amplicons to obtain a protein-coding RNA segment  
18 and the step of translating the RNA into a protein may be carried out by any method known in the  
19 art for expressing a protein from a DNA source. Preferably, the steps of transcription and translation  
20 are carried out in a single reaction vessel (a "single pot" process) in an expression medium that  
21 contains the necessary enzymes and reactants for producing RNA transcripts and for translating the  
22 transcripts into a protein. Kits for *in vitro* transcription and translation of DNA are commercially  
23 available. Commercially available kits include, for example, the Ambion mMessage Machine,  
24 Boehringer Mannheim Linked *in vitro* SP6/T7 Transcription/Translation kit and mammalian  
25 expression systems such as rabbit reticulocytes and canine kidney cells.

26 After the production of a protein, the protein can be separated from the reaction medium by  
27 conventional methods. For example, the proteins may be synthesized as fusions with hexhistidine  
28 moieties, which enable purification by chelation onto nickel columns; or as fusions with numerous  
29 other proteins for which affinity purification methods exist (maltose binding protein, protein A,  
30 protein G, etc). The proteins may also be synthesized using a biotinylated amino acid, which can  
31 facilitate recovery using a streptavidin affinity support. Alternatively, the product protein may be  
32 isolated using HPLC or similar chromatography methods.

1           After the steps of transcription and translation are carried out one time, the solid support  
2 may be reused for subsequent cycles of transcription and translation. If the solid support is in the  
3 form of particles, the particles may be recovered from the reaction medium using separation  
4 techniques including, but not limited to centrifugation, filtration, magnetic separation, or gravity  
5 separation.

6           According to a variation of the method of the present invention, several different proteins  
7 may be produced in one reaction mixture. This may be done by selecting a first PCR primer that  
8 recognizes several different template DNAs, each different template DNA having a coding sequence  
9 for a different protein. The primer would be immobilized onto a solid support as described above  
10 and then, in the amplification step, the solid support would be exposed to the several different  
11 template DNAs, along with soluble primers. PCR amplification would proceed as described above,  
12 and the end result would be that different amplicons (having coding sequences for different proteins)  
13 would all be immobilized on the solid support. The steps of transcription and translation would be  
14 carried out as described above, with the end result that different RNAs would be produced  
15 simultaneously and these would be used to simultaneously produce different proteins. If the  
16 amplicons are designed to include different regulatory sequences controllable by external factors,  
17 the timing and relative amount of each protein that is produced could be controlled.

18           Another way to make different proteins at one time is to immobilize different first PCR  
19 primers that each recognize a different template DNA and then use PCR with different template  
20 DNAs and different second primers to create a solid surface having different amplicons immobilized  
21 on it.

22           Another way to make different proteins at one time is to carry out the immobilization and  
23 amplification steps of the invention separately on different sets of particles with different first PCR  
24 primers and second PCR primers and different template DNAs and then mix the different sets of  
25 particles together before carrying out transcription and translation.

26           In another variation of the present invention, a genetic vaccine or genetic therapeutic may  
27 be created by immobilizing amplicons onto particles, by the method of PCR with an immobilized  
28 primer as described above, combining the particles with a pharmaceutically acceptable carrier and  
29 then injecting the particles into an organism so that the steps of transcription and translation take  
30 place within the organism. In this variation, the template DNA would be selected to produce a  
31 protein antigen or protein therapeutic, and regulatory sequences would be selected so that the  
32 immobilized DNA is expressed within the particular organism. For example, pneumatic epidermal

1 administration, a method that is currently used for injecting microparticles having physisorbed DNA,  
2 may be used with particles of the present invention. An advantage of the present invention is that  
3 the introduction of xenobiotic DNA into a patient is minimized, since selectable markers such as  
4 markers for antibiotic resistance commonly found in bacterial plasmids are not required. Moreover,  
5 pharmaceutical preparations based on the present invention can be predicted to lack pyrogens and  
6 endotoxins, since the protein-encoding DNA is produced chemically and not by way of plasmid  
7 replication inside bacterial cells.

8 Having described the invention, the following examples are given to illustrate specific  
9 applications of the invention, including the best mode now known to perform the invention. These  
10 specific examples are not intended to limit the scope of the invention described in this application.  
11

## 12 SPECIFIC EXAMPLES

### 13 MATERIALS AND METHODS

#### 14 DNA synthesis

15 DNA oligomers were prepared using a PE Applied Biosystems Model 394 DNA/RNA  
16 synthesizer (Foster City, CA) using conventional cyanoethyl (CE) phosphoramidites; an exception  
17 to this is described below under Oligo Affinity Supports. All synthesis chemicals,  
18 phosphoramidites, and chemical modifiers were obtained from Glen Research (Sterling, VA).  
19 Phosphate or disulfide groups were introduced at the 5' -end of oligos using Chemical  
20 Phosphorylation Reagent I or Thiol Modifier C6 S-S, respectively (Glen Research), following the  
21 manufacturer's protocols. Specific details for the coupling of modified oligomers to various  
22 supports are given below. Primers for the bacteriophage T7 promoter region (T7Prom: 5'-TAG-  
23 GGC-GTG-AGT-CGT-ATT- AAA-ATT-AAT-ACG-ACT-CAC-TAT- AGG-GAG-A-3') and T7  
24 termination site (T7Term: 5'-CAA-GGG-GTT-ATG- CTA-GTT-ATT-GCT-CAG-CGG-3') were  
25 prepared and used as either soluble or bead-immobilized PCR primers. The DNA oligomers were  
26 purified and detritylated using C-18 SPE cartridges (Supelco, Bellefonte, PA), and then quantified  
27 as described in Chrisey, L.A., Lee, G.U., and O'Ferrall, C.E. (1996) *Nucl. Acids Res.*, **24**, 3131-  
28 3039. The following Table I details the specific modifications made to these basic sequences.

Table 1. Summary of primer modifications

Primer	5' Chemical Modification	Crosslinker	Bead type	Text Abbreviation
T7Term	None	None	None	T7 Term
T7Term	biotin	None	avidin-MPG	B-MPG
T7Term	phosphorylation	None	EDA-modified-CPG	PC-E
T7Term	phosphorylation	None	HDA-modified-CPG	PC-H
T7Term	C6-thiol	SIAB	EDA-modified-CPG	E-SIAB
T7Term	C6-thiol	SIAB	HDA-modified-CPG	H-SIAB
T7Term	C6-thiol	NHS-PEG-MAL	EDA-modified-CPG	E-PEG
T7Term	C6-thiol	NHS-PEG-MAL	HDA-modified-CPG	H-PEG
T7Term	uncleavable support	None	OAS-PS	T-PS
T7Term	uncleavable support	None	OAS-CPG	T-CPG
T7Prom	None	None	None	T7Prom
T7Prom	uncleavable support	None	OAS-PS	Pr-PS
T7Prom	uncleavable support	None	OAS-CPG	Pr-CPG

## Abbreviations:

T7Prom: PCR primer for T7 promoter region;

T7Term: PCR primer for T7 terminator region;

SIAB: N-succinimidyl-(4-iodoacetyl) aminobenzoate;

NHS-PEG-MAL: N-hydroxysuccinimidyl-polyethylene glycol-maleimide, MW2000;

MPG: Magnetic Porous Glass;

CPG: Controlled-pore Glass;

EDA: N-(2-aminoethyl)-3-aminopropylmethyl-dimethoxy-silane;

HDA: N-(6-aminohexyl)aminopropyl-trimethoxysilane;

OAS, Oligo Affinity Supports;

OAS-PS, polystyrene-polyethylene glycol co-polymer;

OAS-CPG is a commercially available support (Glen Research , Sterling, Va);

1 PC-E: 5'-phosphorylated DNA primers attached to EDA modified CPG by carbodiimide  
2 crosslinking;

3 PC-H: 5'-phosphorylated DNA primers attached to HDA modified CPG by carbodiimide  
4 crosslinking;

5 T-PS: T7Term primers synthesized on OAS-PS supports;

6 T-CPG: T7Term primers synthesized on OAS-CPG supports;

7 Pr-PS: T7Prom primers synthesized on OAS-PS supports;

8 Pr-CPG: T7Prom primers synthesized on OAS-CPG supports;

9 E-SIAB: thiol-modified T7Term primer reacted with EDA modified beads via a SIAB crosslinker;

10 H-SIAB: thiol-modified T7Term primer reacted with HDA modified beads via a SIAB crosslinker;

11 E-PEG: thiol-modified T7Term primer reacted with EDA modified beads via a NHS-PEG-MAL  
12 crosslinker;

13 H-PEG: thiol-modified T7Term primer reacted with HDA modified beads via a NHS-PEG-MAL  
14 crosslinker.

#### 15 **Bead Silanization**

16 As used herein, "dH<sub>2</sub>O" refers to water obtained from a Nanopore purification system which  
17 was >18 MΩ, UV-sterilized and 0.22 μm filtered prior to use. CPG beads (CPG Inc, Lincoln Park,  
18 NJ) having a mean pore diameter of 3.1 μm on particle size of 80/120 mesh, pore volume of 95 cc/g,  
19 and surface area of 8.41 m/g were cleaned by immersion in 1:1 concentrated HCl: MeOH for 45  
20 minutes, rinsed three times with dH<sub>2</sub>O, immersed in concentrated H<sub>2</sub>SO<sub>4</sub> for 45 minutes, and rinsed  
21 in dH<sub>2</sub>O until neutral pH was achieved. The beads were heated in 65°C dH<sub>2</sub>O for 45 minutes prior  
22 to silanization. Beads were then silanized using N-(2-aminoethyl)-3-aminopropylmethyl-dimethoxy-  
23 silane (EDA, Gelest, Tullytown, PA) or N-(6-aminoethyl)-aminopropyltrimethoxysilane (HDA,  
24 Gelest). Acid-cleaned beads (typically 50-100 mg) were shaken with 3-5 mls of a 2% solution of

1 EDA or HDA in 25 mM aqueous  $\text{CH}_3\text{COOH}$  for 1 hour at 25°C. [Care must be taken to avoid  
2 exposure of the stock silanes to moisture. Thus the silanes were opened, dispensed, and re-sealed  
3 in a glovebag under  $\text{N}_2$ ]. The beads were then rinsed 5-10 times with  $\text{dH}_2\text{O}$  (until  $\text{pH} \approx 5$  is attained  
4 for the wash solution), then dried at 120°C to ensure the completion of silane film crosslinking  
5 (dehydration). The dried beads are then ready for use in **Carbodiimide Crosslinking or Treatment**  
6 **with Thiolated DNA** as described below.

#### 7 **Carbodiimide Crosslinking**

8 Reactions containing EDA-modified (15.5 mg, 1 ml) or HDA-modified beads (12.5 mg, 0.8  
9 ml) were incubated on an orbital shaker with 5  $\mu\text{M}$  5'-phosphorylated T7Term and 10 mM 1-ethyl-  
10 3-(3-dimethylamino-propyl)carbodiimide-HCl (EDC, Sigma Chemical Co, St. Louis, MO) in 10  
11 mM 1-methylimidazole (Sigma), pH 7.0, for 3 hours at 50 °C, then 70 hours at 25°C. The beads  
12 were then washed briefly twice with  $\text{dH}_2\text{O}$ , then washed twice with 1 ml of 1M NaCl for 1 hour at  
13 25°C [to remove non-covalently bound DNA]. 5'-phosphorylated DNA primers attached to EDA  
14 or HDA modified CPG by carbodiimide crosslinking are referred to in the text as PC-E and PC-H,  
15 respectively (Table 1).

#### 16 **Treatment with Thiolated DNA**

17 EDA- or HDA-silanized beads (3.5-4.0 mg) were treated with one of two heterobifunctional  
18 crosslinkers: N-succinimidyl-(4-iodoacetyl) aminobenzoate (SIAB; Pierce, Rockford, IL) or N-  
19 hydroxysuccinimidyl-polyethylene glycol-maleimide, MW 2000 (NHS-PEG-MAL; Shearwater  
20 Polymers, Huntsville, AL). Crosslinkers were prepared as a 10 mM solution in 1:4 DMSO:MeOH,  
21 adding the DMSO first to solubilize the crosslinker. Crosslinkers were opened under  $\text{N}_2$  in a  
22 glovebag to minimize hydrolysis of the NHS ester. Beads were agitated with crosslinker solution  
23 (1.5 ml/3.5-4.0 mg beads) for 2.5 hours at 25°C, then rinsed five times with 1 ml portions of MeOH  
24 immediately before coupling to post-PCR thiolated DNA. DNA primers which carried a 5'-disulfide

modifier were used to generate PCR amplicons (see PCR section, below). PCR reactions (100  $\mu$ l) were purified using Qiaquick PCR product purification spin columns (Qiagen, Valencia, CA) yielding 50  $\mu$ l amplicon in Qiagen EB buffer per reaction. For DNA attachment reactions, thiol-modified amplicon was reacted with EDA or HDA modified beads via a SIAB crosslinker (E-SIAB and H-SIAB, respectively) or via a NHS-PEG-MAL crosslinker (E-PEG, H-PEG, respectively). Reactions containing 25  $\mu$ l thiolated amplicon, 1.5 mg beads in HEPES buffer (10 mM HEPES, 1 mM EDTA, pH 6.5), and 200  $\mu$ M tris-(2-carboxyethyl)phosphine (TCEP; Molecular Probes, Eugene, OR) were agitated for 2 hours at 25°C. TCEP rapidly reduces the disulfide modifier on the amplicon to yield free thiol that can react with iodoacetamide or maleimide groups on the crosslinker-modified beads). The beads were then washed with dH<sub>2</sub>O several times, followed by incubation in 1M NaCl for 15 hours to remove non-covalently attached DNA, as described in Chrisey, L.A., Lee, G.U., and O'Ferrall, C.E. (1996) *Nucl. Acids Res.*, **24**, 3131-3039.

### Oligo Affinity Supports (OAS)

Two different types of oligo affinity supports were obtained from Glen Research: OAS-PS (polystyrene-polyethylene glycol co-polymer) and OAS-CPG. These supports were used with 5'-CE-phosphoramidites (Glen Research) to synthesize oligos in the 5' to 3'-direction, which leaves a free OH group at the 3' end (the 5'-end remains attached to the OAS support). The OAS oligos are not susceptible to cleavage during deprotection but remain tethered to the support. T7Term and T7Prom primers synthesized on OAS-PS and OAS-CPG supports are referred to in the text as T-PS or T-CPG and Pr-PS or Pr-CPG, respectively, where T indicates T7Term and Pr indicates T7Prom primers (Table I). The oligo-modified supports were used to prime PCR reactions as described below.

### DNA Templates

Plasmids used as templates in this work included pET3a -derived (Novagen, Madison, WI)



pET23 (described in Andreadis, J.D. and Black, L. (1998) *J. Biol. Chem.*, **273**, 34075-34086) and pET6XGFP (described herein). These plasmids all utilize the bacteriophage T7 expression system, which was selected because of its relatively short (17 base pair) consensus promoter sequence and high level of transcription. pET23 contains a 1.56 kB gene encoding the 57 kDa bacteriophage T4 Major Capsid Protein (T4 MCP). pETGFP was constructed by isolating the *GFP* gene from p6XHis-GFP (Clontech, Palo Alto, California) via PCR and using primer inserted *NdeI* and *BamHI* restriction enzyme sites to clone the *GFP* gene into the pET3a expression vector cleaved with the same restriction enzymes. A third plasmid, pTEI and soluble primers complementary to its sequence were used in amplification experiments designed to quantitate non-specific binding to T7-derived immobilized primers.

#### PCR

The procedure used was a modification of work described in Saiki, R.K., Walsh, P.S., Lenvenson, C.H. and Erlich, H.A. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 6230-6234, and Rasmussen, S.R., Rasmussen, H.B., Larsen, M.R., Hoff-Jorgensen, R., and Cano, R.J. (1994) *Clin. Chem.*, **40**, 200-205. Standard PCR reaction conditions were as follows: 0.1 µg each DNA template (pET6XHISGFP and/or pET23), 1 µM soluble primer A, 50 nM soluble primer B "spike", 30-352 µg bead immobilized primer B (as indicated), 20 µM each dNTP, 0.01% BSA, 0.1% Tween 20, 1 unit of Taq polymerase (Gibco-BRL, Gaithersburg, MD), 10 mM Tris-HCl pH 8.3 at 25°C, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM EDTA and 0.1% gelatin. In this context, primer A refers to unmodified T7Prom or T7Term oligomers and primer B refers to the bead-immobilized version of these primers as described in Table 1. Primer B "spike", refers to the small concentration (50 nM) of soluble primer B added to reactions containing mainly an immobilized form of primer B. The use of a soluble spike primer was critical in the initial stages of PCR amplification to increase the ability of the DNA template to interact with bead-immobilized primers that settled in the bottom

1 of the PCR reaction tube. Control reactions in which neither primer was immobilized, and those in  
2 which a thiolated primer was used for generation of thiolated amplicons, contained 1  $\mu$ m of each  
3 soluble primer.

4 PCR was initiated under hot start and conditions in a PE thermocycler. PCR was conducted  
5 for 35 cycles (each cycle: 1 minute at 94°C, 45 seconds at 66°C, 1 minute at 72°C). The final cycle  
6 was followed by an additional 10 minutes at 72°C to ensure complete extension. For some  
7 experiments, tubes were removed during the denaturation step and vortexed to resuspend the beads.  
8 Subsequent to PCR, the supernatant was removed and beads were washed twice for 30 minutes in  
9 150 mM NaCl (30 minutes each wash) and then in nuclease-free dH<sub>2</sub>O three times (initially for 1  
10 hour, then twice for 15 minutes) with gentle shaking at room temperature. The bead-immobilized  
11 DNA templates were then resuspended in 6  $\mu$ l nuclease-free dH<sub>2</sub>O.

#### 12 **Quantitation of Bead-bound DNA**

13 The amount of 1 kb DNA (pETGFP template) specifically attached to beads during  
14 simultaneous amplification/immobilization was determined using T4 polynucleotide kinase (PNK,  
15 Boehringer Mannheim, Indianapolis, IN) catalyzed incorporation of [ $\gamma$ -<sup>32</sup>P]-dATP (3000Ci/mmol,  
16 NEN Life Science Products, Boston, MA) according to manufacturer's 5'-end-labeling protocols.  
17 PCR with a soluble and a bead-immobilized primer was performed; the soluble control reaction used  
18 a 5'-biotinylated primer and a unmodified primer. After the completion of PCR amplification/  
19 immobilization, soluble amplicon from each PCR reaction was purified via Qiaquick PCR  
20 purification spin columns (Qiagen) and bead-bound amplicon was washed in 150 mM NaCl twice  
21 and rinsed three times with dH<sub>2</sub>O. All samples were resuspended in 25  $\mu$ l dH<sub>2</sub>O final volume. These  
22 reactions were subsequently treated with T4 PNK (37°C, 1 hour, with gentle shaking) and  
23 unincorporated [ $\gamma$ -<sup>32</sup>P]-dATP removed through Qiaquick PCR purification spin columns. Radio-  
24 labeled DNA was then eluted with 50  $\mu$ l dH<sub>2</sub>O. In parallel preparation, bead-bound DNA was treated

1 with T4 PNK, under the same conditions described above, and washed twice with 150 mM NaCl and  
2 rinsed three times with dH<sub>2</sub>O. Samples were placed in 10 ml Ecoscint H (National Diagnostics,  
3 Atlanta, GA) and a Packard 1500 Liquid Scintillation Counter (Packard, Downers Grove, IL) was  
4 utilized to determine [ $\gamma$ -<sup>32</sup>P]-dATP incorporation in the samples. Non-specific binding to CPG or  
5 PS beads was determined by 5' end-labeling a target gene (pTEI template, target gene approximately  
6 0.7 kb), whose sequence was non-complementary to the T7-derived immobilized primer. The  
7 quantity of both soluble and bead-immobilized DNA bound, respectively, was calculated using the  
8 specific activity of the [ $\gamma$ -<sup>32</sup>P]-dATP. The percentage of total amplicon immobilized during solid-  
9 phase PCR was determined as the ratio of bead-associated cpm to the total cpm (cpm of bead-bound  
10 DNA plus cpm of soluble DNA from a given PCR reaction). The data reported are the result of  
11 triplicate experiments.

#### 12 ***In vitro* transcription/translation reactions**

13 Boehringer Mannheim (Indianapolis, IN) Linked *in vitro* SP6/T7 - Transcription/Translation  
14 Kit (nonradioactive) was used, according to manufacturer protocols, to obtain mRNA transcripts and  
15 protein products using soluble or bead-immobilized DNA. Ambion (Austin, TX) mMessage  
16 mMachine *in vitro* transcription system was used to determine whether bead-immobilized DNA  
17 templates could be recycled or reused. Briefly, DNA immobilized templates were incubated with  
18 transcription reagents for 80 minutes at 37°C with periodic gentle shaking or continuous agitation.  
19 Samples were pelleted briefly (700 x g, 2 minutes, 25°C) and the mRNA transcript-containing  
20 supernatant was removed and analyzed electrophoretically (see **Electrophoretic analysis**). Bead-  
21 immobilized DNA templates were then washed twice with 150 mM NaCl and twice with nuclease-  
22 free dH<sub>2</sub>O (30 minutes each with gentle shaking) and fresh transcription reaction reagents added.  
23 This was repeated for a total of seven cycles.

## Electrophoretic analysis

mRNA transcripts were analyzed on 1-2% agarose gels prepared in 1X MOPS and 6% formaldehyde under RNase-free conditions and electrophoresed at 100V (100-150 mA) for 1 - 1.5 hours, as described in Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) *Molecular Cloning: a Laboratory Manual*, 1st ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp 7.43 - 7.45. RNA was visualized using ethidium bromide and photographed under UV light. Relative band intensities of the experimental conditions to the control transcript band were determined by scanning the photo into CorelDraw 7 (Corel Corporation) and using NIH Image 1.61 (W. Rasband, NIH, Bethesda, MD). For Western blot analysis, protein products were electrophoresed in 12% precast Novex (San Diego, CA) acrylamide gels, electroblotted onto Immobilon-P membranes (Millipore, Billerica, MA), and probed with a 1:1000 dilution of streptavidin-horseradish peroxidase (HRP) conjugate (GIBCO-BRL, Gaithersburg, MD). Stabilized TMB substrate (Promega, WI) was used to visualize the biotinylated protein-streptavidin-HRP complexes.

## RESULTS AND DISCUSSION

### 1. Immobilization of DNA primers

Primers synthesized on OAS supports linked to PS or CPG beads were synthesized in the 5' to 3' direction and remained attached to the PS or CPG support at the 5'-end. Thus, primers synthesized using OAS supports can be used directly as immobilized primers in PCR reactions without further manipulation. A potential disadvantage to the use of OAS supports is an inability to remove failure sequences generated during DNA synthesis. All other primers were purified after synthesis before coupling to beads, to remove failure sequences.

In order to be useful for a PCR reaction, bead-bound primers must remain intact during the

1 thermal cycling process. Initially, 5'-thiolated primers attached via heterobifunctional crosslinkers  
2 to amine-functionalized CPG were tested, and as expected, and as described in Chrisey, L.A., Lee,  
3 G.U., and O'Ferrall, C.E. (1996) *Nucl. Acids Res.*, **24**, 3131-3039, these proved to be heat-labile  
4 (data not shown). Thus, a soluble 5-disulfide-modified primer was used to generate thiolated PCR  
5 product and immobilization took place as a separate step, subsequent to amplification. The  
6 remainder of the immobilized primer chemistries tested were heat stable and thus suitable for solid-  
7 phase PCR experiments. **Table 1** summarizes the various combinations of primers, attachment  
8 chemistries and beads or polymeric supports that were used in this example.

9 Several amplification conditions were analyzed for the generation of a bead-bound DNA  
10 template that was functional for transcription by the bacteriophage T7 polymerase. The primers  
11 used in the amplification reactions were as follows (see **Table 1**): 1) soluble T7 Prom and soluble  
12 T7Term, 2) soluble T7Prom and soluble 5'-thiolated T7Term, 3) soluble T7Prom and immobilized  
13 T7Term (PC-E, PC-H, T-PS, or T-CPG), and 4) immobilized T7 promoter primer (Pr-PS or Pr-CPG)  
14 and soluble T7Term. All PCR and transcription reactions yielded soluble DNA or RNA fragments  
15 of the expected size as verified by electrophoretic analysis.

16 The amount of DNA covalently bound to the CPG (PC-E or PC-H), OAS-CPG (T-CPG or  
17 Pr-CPG) or PS (T-PS or Pr-PS) beads was determined by 5'-end radiolabeling DNA amplicons  
18 obtained following PCR with or without an immobilized primer. A 5' biotin labeled primer was  
19 used as one of two primers in the soluble control reaction to limit radiolabeling to just one of the 5'  
20 ends of the amplicon and thus, to more closely simulate the situation in which one primer is bead  
21 bound. Briefly, the gene for GFP was amplified from pET6XGFP using the following conditions:  
22 1) two soluble primers (T7Prom and 5'-biotinylated T7Term), 2) one soluble and one 5'-thiol-  
23 modified primer (T7Prom and 5'SH-modified T7Term) or 3) one soluble and one immobilized  
24 primer (T7Term and PC-E, PC-H, T-PS, or T-CPG) or (Pr-PS or Pr-CPG and T7Term). The

thiolated PCR amplicon generated under condition number 2 above was then immobilized to heterobifunctional crosslinker-modified CPG beads. All bead immobilized templates and soluble PCR fractions were 5'-end labeled as described above. Nonspecific DNA attachment was determined by amplifying a DNA template without any complementarity to the above soluble or immobilized primers, and attempting to radiolabel bead-associated product. Specific covalent attachment to the immobilized primers was determined by the amount of  $^{32}\text{P}$  incorporation associated with the beads minus any nonspecific attachment. The specific activity of the  $[\gamma^{32}\text{P}]$ -dATP was used to determine the nmol DNA bound per gram beads. **Table 2** compares the yield of DNA PCR amplicons on CPG, OAS-CPG, or OAS-PS beads using each type of immobilized primer, or post-PCR attachment of thiolated amplicons.

**Table 2.** Quantitation of bead-immobilized DNA

Primer used for immobilization of amplicon	nmol DNA/g bead specific binding
PC-E	141 $\pm$ 0.48
PC-H	374 $\pm$ 0.39
5'-thiol modified T7Term ( $\Rightarrow$ E-SIAB) <sup>a</sup>	77 $\pm$ 0.75
5'-thiol modified T7Term ( $\Rightarrow$ H-SIAB) <sup>a</sup>	154 $\pm$ 2.5
5'-thiol modified T7Term ( $\Rightarrow$ E-PEG) <sup>a</sup>	15.4 $\pm$ 0.63
5'-thiol modified T7Term ( $\Rightarrow$ H-PEG) <sup>a</sup>	75.3 $\pm$ 0.28
T-PS	25.2 $\pm$ 0.6
T-CPG	10.8 $\pm$ 0.28
P-PS	18.9 $\pm$ 0.84
P-CPG	41.8 $\pm$ 1.2

Experimental detail is given in the **MATERIALS AND METHODS** section. Bead-bound primers used during the PCR amplification/immobilization protocol are indicated in the far left column using abbreviations described in Table I legend. After PCR amplification, soluble and immobilized DNA were purified separately and labeled with  $[\gamma^{32}\text{P}]$ -dATP using T4 polynucleotide kinase at 37°C for one hour. Bound DNA was determined by the specific activities of the oligomers. Non-specific

1 binding was determined by PCR amplification of an approximately 1 kb target gene, whose sequence  
2 is non-compatible to the bead-bound primer. <sup>a</sup>The 5'-thiol-modified T7Term primers were used to  
3 generate soluble PCR amplicons which were subsequently crosslinked to CPG beads via the  
4 chemistry indicated in parentheses. The total soluble thiolated PCR product and the immobilized  
5 DNA was radiolabeled as described above to quantitate the amount of PCR product immobilized  
6 versus total soluble PCR product. Amplification of a 1 kb target sequence, compatible to the soluble  
7 and bead-bound primer, was used to determine specific binding. The data are reported as the mean  
8 of triplicate experiments  $\pm$  standard deviation. Nonspecific binding is calculated as the total binding  
9 minus non-specific binding for a particular immobilized primer.

10 The results indicate a wide range of DNA attachment in the nmol DNA/gram bead range for  
11 the immobilization chemistries surveyed. It is interesting to note that HDA silanized beads (H-  
12 SIAB, H-PEG, and PC-H) consistently yielded higher quantities of covalently attached DNA than  
13 EDA silanized beads for all three chemical attachment methods tested. HDA (6 carbons) is a longer  
14 silane than EDA (3 carbons) and may be less densely packed on the surface than the EDA. A lower  
15 density of silane may provide a greater proportion of silanes that react with the heterobifunctional  
16 crosslinkers. DNA template attachment is most successful on beads to which primers were coupled  
17 prior to PCR via carbodiimide chemistry (PC-E and PC-H) and those that underwent post PCR  
18 immobilization onto HDA crosslinker modified CPG (see PC-E, PC-H, H-SIAB, H-PEG, Table 2).

19 The percentage of total PCR product that is bound to the immobilized beads during solid-  
20 phase PCR is shown in the following Table 3.

**Table 3. Percentage of total PCR amplicon immobilized**

Solid-phase primer	Percent of total
Non-specific PC	0.45 $\pm$ 0.25
PC-E	30.9 $\pm$ 0.73
PC-H	60.4 $\pm$ 2.2
Non-specific OAS-PS	0.44 $\pm$ 0.16
P-PS	3.21 $\pm$ 0.54
T-PS	51.8 $\pm$ 2.7
Non-specific OAS-CPG	0.38 $\pm$ 0.08
P-CPG	3.04 $\pm$ 0.53
T-CPG	16.7 $\pm$ 3.5

Experimental detail is given in **MATERIALS AND METHODS**, above. Non-specific PC, PS, and CPG refer to non-specific binding to those specific beads. Percentage of total PCR amplicon immobilized was determined by dividing bead bound cpm by total cpm (bead bound cpm and soluble DNA cpm) and multiplying by 100%. Experiments were conducted in triplicate and the data are given as the mean  $\pm$  the standard error of the mean.

As shown in Table 3, the mean bead-bound amplicon generated by PC-E and PC-H immobilized primers (expressed as a percentage of total amplicon) was  $30.9 \pm 0.73\%$  and  $60.4 \pm 2.2\%$ , respectively. Amplification using T-PS and T-CPG solid-phase primers resulted in  $51.8 \pm 2.7\%$  and  $15.6 \pm 3.5\%$  of total amplicon as bound to PS and CPG beads, respectively. Analysis of the four OAS supports indicated a comparatively low mean percentage of total amplicon bound to P-PS and P-CPG immobilized primers ( $3.2 \pm 0.54\%$  and  $3.0 \pm 0.53\%$ , respectively). This result may be sequence-dependent as these primer-bead combinations used the T7Prom primer, which contains a potential hairpin loop sequence that may have interfered with the annealing of the primer to the target molecule.

PCR reactions that contained soluble T7Prom and immobilized T7Term primers, but no soluble "spike" of T7Term primer, resulted in undetectable soluble PCR product formation. The



1 addition of a "spike" of soluble T7Term primer was necessary to increase the likelihood of an  
2 interaction between soluble coding strand and the bead-bound T7Term primer that settles to the  
3 bottom of the PCR tube during amplification. To ascertain whether settlement of the beads during  
4 PCR was inhibiting efficient coding strand capture, select reactions were repeated, but the protocol  
5 was altered such that the reaction tubes were vortexed vigorously during the denaturation stage of  
6 several cycles (2,4,6,8,12, and 24). Contrary to expectations, this resulted in a diminished yield  
7 of lower quality transcript (which reached a maximum of 66% of the non-vortexed reaction).

8 All bead-immobilized DNA's tested were functional as templates for *in vitro* transcription.  
9 Following the PCR reactions, the 1.56 kB gene encoding the T4 MCP attached to PC-E and PC-H  
10 beads were subjected to *in vitro* transcription. Electrophoretic analysis of the mRNA transcripts  
11 produced from these reactions shows that although mRNA is produced with each bead-type, PC-E  
12 immobilized PCR amplicon is more readily transcribed than DNA attached through PC-H primers.  
13 This result was also consistent with *in vitro* transcription results for amplicons produced from 5'  
14 thiol-modified primers and subsequently attached to EDA or HDA-modified CPG beads (E-SIAB,  
15 H-SIAB, E-PEG, and H-PEG) in that more mRNA was produced when EDA-silanized beads were  
16 used. Paradoxically, the observed higher levels of amplicon attachment to HDA silanized beads may  
17 be detrimental to efficient *in vitro* transcription of the immobilized template DNA. This may be due  
18 to the inability of T7 polymerase and other required transcriptional components to efficiently access  
19 and transcribe because of tight DNA packing on and inside the CPG bead pores.

20 A range of concentrations of bead-bound DNA template were examined to establish whether  
21 or not a linear relationship exists between the quantity of bead-bound DNA template added to  
22 transcription reactions and mRNA produced. A comparison of band intensities of mRNA  
23 transcribed from bead-immobilized DNA versus soluble DNA template showed a negligible  
24 difference in transcript production above 62 µg beads. The results relative to the *Xenopus* control

1 (1.00) were 0.65, 0.80, 0.84 for 10, 62, and 124  $\mu$ g PC-E immobilized DNA, respectively. The  
2 results for PC-H relative to the same control are 0.32, 0.28, 0.32 for 10, 62, and 124  $\mu$ g beads. These  
3 observations indicated a near saturated state with little change with increasing bead-bound amplicon  
4 concentration. For nearly all of the transcription experiments, reactions were periodically gently  
5 agitated during incubation. To determine whether the observed limitation in product formation was  
6 due to the restricted accessibility of the immobilized DNA by the transcription reactants as the beads  
7 settled to the bottom of the reaction tube, we repeated select experiments and continually agitated  
8 during the incubation period. This resulted in an average increase of 19.5% product mRNA (when  
9 greater than 50  $\mu$ g of beads were used per transcription reaction), verifying that exposure of the  
10 beads to fresh transcription reagents through mixing improves yield.

11 It is important to note that the relative intensity of the mRNA bands were approximately 80%  
12 of the soluble *Xenopus* DNA transcriptional control, demonstrating the efficiency of the solid-phase  
13 templates in *in vitro* transcription reactions.

14 A primary interest in developing a solid-phase PCR amplification system using bead-bound  
15 primers was for their potential use in repetitive *in vitro* transcription reactions. *In vivo* protein  
16 synthesis of polymeric proteins and certain mammalian proteins can be problematic due to the  
17 rearrangement or modification of highly repetitive DNA sequences, improper protein folding, and  
18 cellular toxicity. Although *in vitro* transcription/translation systems could provide an alternative to  
19 *in vivo* protein synthesis, these methodologies can be impractical because of the laborious and often  
20 expensive necessity to resynthesize DNA templates after each transcription reaction. Conversely,  
21 automated solid-phase peptide synthesis can be quite economical but may be limited by the length  
22 and sometimes the sequences of the desired protein. Bead-immobilized DNA templates would  
23 provide a convenient way to circumvent these problems by enabling the bead-bound DNA templates  
24 to be collected and recycled after each transcription reaction. This approach permits the synthesis

1 of large proteins not available by automated synthesis and is more economical than conventional *in*  
2 *vitro* transcription/translation reactions. Experiments were carried out to determine whether DNA  
3 templates, coding for GFP (1.0 kB), immobilized via PC-E and E-PEG crosslinked supports, could  
4 be efficient templates for multiple sequential *in vitro* transcription reactions. In these experiments,  
5 bead-immobilized PCR amplicons used as templates for *in vitro* transcription, then the immobilized  
6 DNA primers were collected by brief centrifugation, rewashed, and exposed to fresh *in vitro*  
7 transcription reagents. Significant differences in sequential mRNA production were observed for  
8 each type of immobilized DNA template. Although E-PEG yielded high levels of transcript initially,  
9 these templates were unable to survive the recycling process beyond 2-3 cycles of repetitive rounds  
10 of *in vitro* transcription. This result proved to be the case for most immobilized DNA templates  
11 tested with the exception of PC-E immobilized DNA. In this case, the findings indicated that PC-E  
12 bound templates can be collected and recycled effectively for use in at least seven repetitive rounds  
13 of *in vitro* transcription.

## 14 2. Protein production from solid-phase DNA templates

15 To determine whether mRNA transcripts produced from solid-phase DNA templates were  
16 able to initiate translation, reagents for non-radioactive *in vitro* translation were added to the  
17 supernatants from *in vitro* transcription reactions. The biotinylated protein products were detected  
18 using a streptavidin-HRP conjugate with TMB substrate. A Western blot of biotinylated products  
19 produced from solid-phase templates through a coupled *in vitro* transcription/translation system  
20 showed that all solid-phase DNA templates produced the desired protein product (57 kDa T4 MCP).  
21 A useful feature of the bead immobilization methodology is the ability to amplify and to immobilize  
22 either single or multiple DNA target sequences. In a mixing experiment, the genes for GFP and the  
23 T4 MCP were amplified via solid-phase PCR using the immobilized PC-E primer and pET6XGFP  
24 and pET23 plasmids as templates (either separately or mixed together). The bead immobilized

1 amplicons from each solid-phase PCR reaction (GFP, MCP, GFP + MCP) were collected, washed,  
2 and used as templates for *in vitro* transcription/translation reactions. A Western Blot showed the  
3 separate production of GFP and the T4 MCP and *in vitro* translation of both proteins simultaneously  
4 from a mixture of immobilized templates. Thus, the addition of multiple DNA gene templates to  
5 solid-phase PCR reactions yields efficient immobilization of both genes and the subsequent  
6 production of multiple protein products.

7 Obviously, many modifications and variations of the present invention are possible in light  
8 of the above teachings. It is therefore to be understood that, within the scope of the appended claims,  
9 the invention may be practiced otherwise than as specifically described.

### Claims

What is claimed is:

1. A method of making a protein, the method comprising the steps of:

(a) providing

(i) a solid support,

(ii) a template DNA that includes at least one sequence that encodes a protein, and

(iii) first and second oligonucleotide primers, wherein the first oligonucleotide primer, the second oligonucleotide primer and/or the template DNA include at least one transcription regulatory sequence and at least one translation regulatory sequence, and wherein the first oligonucleotide primer is covalently attached to the solid support,

(b) amplifying the template DNA using the first and second oligonucleotide primers to obtain amplicons covalently attached to the solid support, wherein the amplicons include the transcription and translation regulatory sequences and the protein coding sequence,

(c) transcribing the covalently attached amplicons to obtain an RNA segment that includes the protein coding sequence, and

(d) translating the RNA segment to obtain the protein.

2. A method of making a protein, the method comprising the steps of:

(a) providing

(i) a solid support,

(ii) a template DNA that includes at least one sequence that encodes a protein, and

(iii) first and second PCR primers, wherein the first PCR primer, the second PCR

primer and/or the template DNA include at least one transcription regulatory sequence and at least one translation regulatory sequence, and wherein the first PCR primer is covalently attached to the solid support,

(b) amplifying the template DNA by the polymerase chain reaction using the first and second PCR primers to obtain amplicons covalently attached to the solid support, wherein the amplicons include the transcription and translation regulatory sequences and the protein coding sequence,

(c) transcribing the covalently attached amplicons to obtain an RNA segment that includes the protein coding sequence, and

(d) translating the RNA segment to obtain the protein.

3. The method of Claim 2 wherein the solid support comprises at least one particle.

4. The method of Claim 2 wherein the solid support comprises at least one particle selected from the group consisting of glass particles, controlled porous glass particles, magnetic particles, polymeric particles, hydrogel particles, sol-gel particles and ceramic particles.

5. The method of Claim 3 wherein the solid support comprises at least one particle having a particle size of about 50 nanometers to 10 mm along an axis of greatest length.

6. The method of Claim 2 wherein the step (a) of providing a solid support having the first PCR primer covalently attached thereto is accomplished by synthesizing the first PCR primer onto the solid support.

7. The method of Claim 2 wherein the solid support has a surface that has amine groups, wherein the first PCR primer has phosphate groups, and wherein step (a) of providing a solid support having the first PCR primer covalently attached thereto is accomplished through carbodiimide coupling of the amine groups of the surface of the solid support to the phosphate groups of the first PCR primer.
8. The method of Claim 2 wherein the solid support has a surface that has amine groups, wherein the first PCR primer has amine groups, and wherein step (a) of providing a solid support having the first PCR primer covalently attached thereto is accomplished through carbodiimide coupling of the amine groups of the surface of the solid support to the amine groups of the first PCR primer.
9. The method of Claim 2 wherein the first PCR primer has an amine group and wherein step (a) of providing a solid support having the first PCR primer covalently attached thereto is accomplished by means of a homobifunctional crosslinker that covalently attaches to the solid support and that has an amine-reactive group that reacts with the amine group of the first PCR primer.
10. The method of Claim 2 wherein the first PCR primer has an amine group and wherein step (a) of providing a solid support having the first PCR primer covalently attached thereto is accomplished by means of a heterobifunctional crosslinker that covalently attaches to the solid support and that has an amine-reactive group that reacts with the amine group of the first PCR primer.
11. The method of Claim 2 wherein the first PCR primer is between 5 and 500 bases long.
12. The method of Claim 2 wherein the first PCR primer includes regulatory sequences from bacterial, viral, fungal, mammalian or insect cell expression systems.

13. The method of Claim 2 wherein the first PCR primer includes a T7 transcription promoter sequence.

14. The method of Claim 2 wherein at the conclusion of step (d), the solid support is separated from the protein produced in step(d), and steps (c) and (d) are repeated at least one additional time.

15. A method of making a protein, the method comprising the steps of:

(a) providing

(i) a solid support,

(ii) a template DNA that includes at least one sequence that encodes a protein, and

(iii) first and second PCR primers, wherein the first PCR primer, the second PCR primer and/or the template DNA include at least one transcription regulatory sequence and at least one translation regulatory sequence,

(b) amplifying the template DNA by the polymerase chain reaction using the first and second PCR primers to obtain amplicons that include the transcription and translation regulatory sequences and the protein coding sequence,

(c) covalently attaching the amplicons to the solid support,

(d) transcribing the covalently attached amplicons to obtain an RNA segment that includes the protein coding sequence, and

(e) translating the RNA segment to obtain the protein.

16. A method of making a plurality of different proteins simultaneously, the method comprising the steps of:

(a) providing



- (i) a solid support,
  - (ii) a template DNA that includes a plurality of different coding sequence that each encodes a different protein, and
  - (iii) first and second PCR primers, wherein the first PCR primer, the second PCR primer and/or the template DNA include at least one transcription regulatory sequence and at least one translation regulatory sequence, and wherein the first PCR primer is covalently attached to the solid support,
- (b) amplifying the template DNA by the polymerase chain reaction using the first and second PCR primers to obtain amplicons covalently attached to the solid support, wherein the amplicons includes a transcription regulatory sequence and the plurality of different protein coding sequence,
- (c) transcribing the covalently attached amplicons to obtain a plurality of different RNA segments that each include a protein coding sequence, and
- (d) translating the plurality of different RNA segments to obtain a plurality of different proteins.

17. A method of making a plurality of different proteins simultaneously, the method comprising the steps of:

- (a) providing
  - (i) a solid support,
  - (ii) a plurality of different template DNAs, each including a sequence that encodes a different protein, and
  - (iii) a first PCR primer that is covalently attached to the solid support and that hybridizes with each of the different template DNAs, and

- (iv) a plurality of different second PCR primers, each hybridizing with a different template DNA,  
wherein either the first PCR primer or the second PCR primers or the template DNA includes at least one transcription regulatory sequence and at least one translation regulatory sequence,
- (b) amplifying the plurality of different template DNA by the polymerase chain reaction using the first and second PCR primers to obtain a plurality of different amplicons covalently attached to the solid support, wherein each amplicons includes a transcription regulatory sequence and a protein coding sequence,
- (c) transcribing the plurality of different covalently attached amplicons to obtain a plurality of different RNA segments that each include a protein coding sequence, and
- (d) translating the plurality of different RNA segments to obtain a plurality of different proteins.

18. A method of preparing and delivering DNA to an organism, wherein the DNA has at least one protein encoding sequence, at least one transcription regulatory sequence and at least one translation regulatory sequence, the method comprising the steps of:

- (a) providing
  - (i) a solid support, wherein the solid support comprises particles having a size sufficiently small to be injected into an organism,
  - (ii) a template DNA that includes at least one sequence that encodes a protein, and
  - (iii) first and second oligonucleotide primers, wherein the first oligonucleotide primer, the second oligonucleotide primer and/or the template DNA include at least one transcription regulatory sequence and at least one translation regulatory

sequence, and wherein molecules of the first oligonucleotide primer are covalently attached to the particles,

- (b) amplifying the template DNA using the first and second oligonucleotide primers to obtain amplicons covalently attached to the particles, wherein the amplicons include the transcription and translation regulatory sequences and the protein coding sequence,
- (c) combining the particles having covalently attached amplicons with a pharmaceutically acceptable carrier, and
- (d) injecting the particles into an organism.

19. A method of preparing and delivering DNA to an organism, wherein the DNA has at least one protein encoding sequence, at least one transcription regulatory sequence and at least one translation regulatory sequence, the method comprising the steps of:

(a) providing

- (i) a solid support, wherein the solid support comprises particles having a size sufficiently small to be injected into an organism,
- (ii) a template DNA that includes at least one sequence that encodes a protein, and
- (iii) first and second PCR primers, wherein the first PCR primer, the second PCR primer and/or the template DNA include at least one transcription regulatory sequence and at least one translation regulatory sequence, and wherein molecules of the first PCR primer are covalently attached to the particles,

(b) amplifying the template DNA by the polymerase chain reaction using the first and second PCR primers to obtain amplicons covalently attached to the particles, wherein the amplicons include the transcription and translation regulatory sequences and the protein coding sequence,

- (c) combining the particles having covalently attached amplicons with a pharmaceutically acceptable carrier, and
- (d) injecting the particles into an organism.

20. A device for making a protein, the device comprising

- (i) a solid support, and
- (ii) a DNA segment having a first end and a second end, wherein the first end is covalently attached to the solid support, wherein the second end contains phosphorothioate linkages, and wherein the DNA segment includes at least one protein coding sequence, at least one transcription regulatory sequence and at least one translation regulatory sequence.

21. A pharmaceutical composition comprising

- (i) a plurality of particles, the particles having a size sufficiently small to be injected into an organism,
- (ii) a plurality of molecules of a DNA segment, wherein the DNA segment has a first end and a second end, wherein the first end is covalently attached to the particles, wherein the second end contains phosphorothioate linkages, and wherein the DNA segment includes at least one protein coding sequence, at least one transcription regulatory sequence and at least one translation regulatory sequence, and
- (iii) a pharmaceutically acceptable carrier.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/34426**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : C12Q 1/68; A61K 48/00; C07H 21/04; C12P 19/34

US CL : 435/6, 69.1, 91.1, 91.3; 514/2, 44; 536/23.1, 25.3

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1, 91.1, 91.3; 514/2, 44; 536/23.1, 25.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, MEDLINE, EMBASE, CAPLUS, SCISEARCH

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,571,690 A (HECHT et al.) 05 November 1996, see entire document.	1-21
Y	US 5,665,563 A (BECKLER) 09 September 1997, see entire document.	1-21
Y	US 5,700,667 A (MARBLE et al.) 23 December 1997, see entire document.	1-21
Y	BEATTIE et al. Hybridization of DNA Targets to Glass-Tethered Oligonucleotide Probes. Molecular Biotechnology. 1995, vol. 4, pages 213-225, see entire document.	1-21

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*I* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

23 MARCH 2001

Date of mailing of the international search report

01 JUN 2001

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/34426

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	ANDREADIS et al. Use of immobilized PCR primers to generate covalently immobilized DNAs for in vitro transcription, translation reactions. Nucleic Acids Research. 2000, vol. 28, no 2, pages i-viii, see entire document.	1-21
Y	MARBLE et al. RNA Transcription from Immobilized DNA Templates. Biotechnol. Prog. 1995, vol. 11, pages 393-396, see entire document.	1-21